POLY(ADP-RIBOSE) POLYMERASE ACTIVITY IN NUCLEOTIDE PERMEABLE CELLS

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Received 6 October 1977

1. Introduction

Poly(ADP-ribose) polymerase is a chromatin-bound enzyme which catalyzes the transfer of (ADP-ribose) units from NAD^+ to chromatin proteins to form poly(ADP-ribose). The enzyme activity is dependent on DNA and is stimulated by histones. Although the precise function of poly(ADP-ribose) polymerase is unknown, its chromatin location, its dependence on DNA and some indirect evidence suggest that it is related to nuclear activities (reviewed [1,2]). Enzyme assays have generally been performed with isolated nuclei because the specific substrate, NAD^+, does not permeate intact, living cells.

DNA synthesis in isolated nuclei is often a poor reflection of the in vivo activity, and we have been concerned that the same might be true for poly(ADP-ribose) synthesis. The apparent activity of poly(ADP-ribose) polymerase may be dependent on the state of the chromatin. In that case it would obviously be important to measure the enzyme in a system where the chromatin is damaged as little as possible.

In this paper we describe the activity of poly(ADP-ribose) polymerase in mouse lymphoma L5178Y cells rendered permeable to nucleotides by treatment with hypotonic buffer. DNA synthesis in permeabilized cells is a semiconservative continuation of the in vivo synthesis [3]. Using this system the activity of poly-(ADP-ribose) polymerase is very low. Our results indicate that the reported high activity in isolated nuclei is an artifact of the nuclear isolation procedure. It is shown that the high enzyme activity is associated with fragmentation of the DNA; it is argued that an approximation of the in vivo poly(ADP-ribose) polymerase activity requires at least the demonstration that the DNA has not decreased in size; and it is shown that this is so in these permeabilized cells.

2. Materials and methods

2.1. *Cell line*

Mouse lymphoma L5178Y cells were grown in suspension culture in Fischer's medium supplemented with 10% horse serum, penicillin (50 units/ml, 30 μg/ml) streptomycin (50 μg/ml) and sodium pyruvate (200 μg/ml).

2.2. *Preparation of permeabilized cells*

The modification [3] of the procedure [4] was used. Cells were cooled to 0°C, washed with Pucks saline A (5.4 mM KCl (400 mg/litre), 137 mM NaCl (8 g/litre), 4.2 mM $NaHCO_3$ (350 mg/litre) and 5.5 mM glucose (1 g/litre)) and resuspended in hypotonic solution at $2–3 \times 10^7$ cells/ml for permeabilization. The hypotonic solution contained 9 mM hepes pH 7.8, 5 mM dithiothreitol, 4.5% (w/v) dextran approx. mol. wt 110 000, 1 mM EGTA, 4.5 mM $MgCl_2$. After 30 min a 9-fold vol. isotonic buffer solution was added giving a final cell density of $2–3 \times 10^6$ cells/ml. The isotonic buffer solution contained 40 mM hepes pH 7.8, 130 mM KCl, 2.5 mM dithiothreitol, 4% (w/v) dextran, 225 mM sucrose, 2 mM EGTA, 2.3 mM $MgCl_2$.

2.3. *Poly(ADP-ribose) polymerase assay*

The poly(ADP-ribose)polymerase assay was performed in the isotonic buffer solution. Cells, 300 μl, or nuclei were incubated at 26°C with 50 μl of [^{3}H]-NAD^+, final concentration 1.4 μCi/ml, 70 μM. At

various times, 2 ml 10% (w/v) trichloroacetic acid were added to duplicate samples. After 30 min or more at 0°C the precipitated cells were filtered onto glass fibre discs, washed 6 times with 1% trichloroacetic acid, once with acetone and the radioactivity counted. The radioactive trichloroacetic acid-insoluble material was shown to be poly(ADP-ribose) by digestion with snake venom phosphodiesterase and thin layer chromatography of the products [5].

2.4. *Sucrose density centrifugation*

Cells were prelabelled overnight with [^{3}H]thymidine (1.0 μCi/ml, approx. spec. act. 20 Ci/mmol) in the presence of 1.0 μM nonradioactive thymidine. Alkaline sucrose gradients containing 4.7 ml of 5–20% sucrose with 100 mM NaOH and 100 mM NaCl were overlayed with 200 μl 2% (w/v) SDS, 20 mM EDTA. A 1:10 diluted cell suspension (100 μl, 2–3 × 10^4 cells) was pipetted onto the detergent layer on the gradients which were then centrifuged at 25 000 rev/min for 60 min at 20°C in a SW 50.1 rotor in a Beckman L5.65 centrifuge. After centrifugation 16 drop fractions were collected onto Whatman grade 17 paper strips and the trichloroacetic acid insoluble radioactivity of each fraction counted.

2.5. *Isolation of nuclei*

Nuclei were isolated by the method in [6]. Cells were washed in buffered, 150 mM sucrose and resuspended in this solution. A detergent solution containing sodium deoxycholate and Nonidet P40 was added and the mixture shaken by hand for 5 min. The resulting nuclei were washed and resuspended at a density of 2–3 × 10^6 nuclei/ml in the isotonic buffer.

3. Results and discussion

Figure 1 compares the activity of poly(ADP-ribose) polymerase in permeabilized cells and in isolated nuclei. In permeabilized cells the initial rate of incorporation is very low (3–4 pmol/min/10^6 cells) compared to that in nuclei (30–40 pmol/min/10^6 nuclei). However, the enzyme rate in the permeabilized cells increases upon incubation at 26°C. This increase depends on the time of incubation as shown in fig.2. Cells were pre-incubated at 26°C without NAD^+ for different lengths of time and then the rate of formation of polymer was measured by adding radioactive NAD^+. The enzyme activity without pre-incubation is low and increases linearly with the duration of pre-incubation.

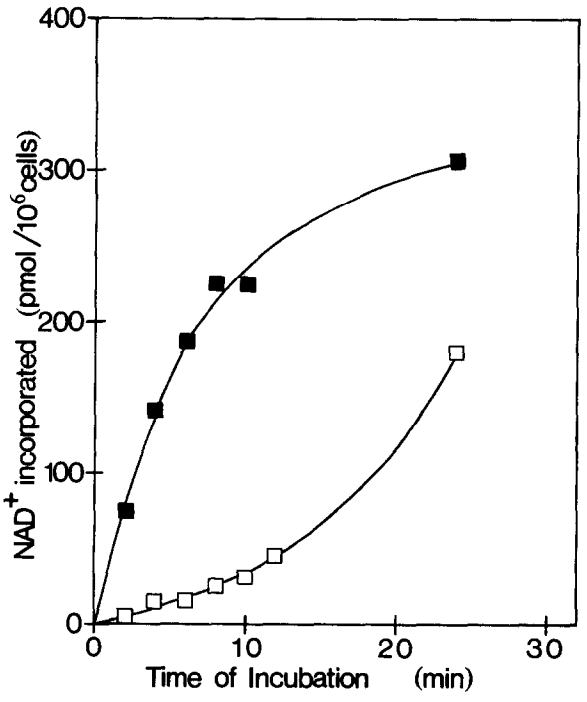

Fig.1. Comparison of the enzyme activity of poly (ADP-ribose) polymerase in hypotonically permeabilized cells (□) and in isolated nuclei (■).

The initial reason for using permeabilized cells was that we supposed that their chromatin would be less damaged than that of isolated nuclei. We therefore measured DNA size by alkaline sucrose gradient analysis. The size of the DNA in permeabilized cells is indistinguishable from that in intact cells, whereas the DNA in nuclei is fragmented (fig.3A). In this experiment all three samples were exposed to 500 rad γ-radiation immediately prior to loading onto the gradient [7]. This confirms the observation that the DNA is not fragmented in the permeabilized cells [3].

However, during incubation at 26°C in isotonic buffer, the DNA of the permeabilized cells is degraded to smaller sizes (fig.3B). The activity of poly(ADP-ribose) polymerase is increased at the same time (fig.2).

Incubation of the permeabilized cells at 26°C for 90 min in the presence of the complete DNA synthesis

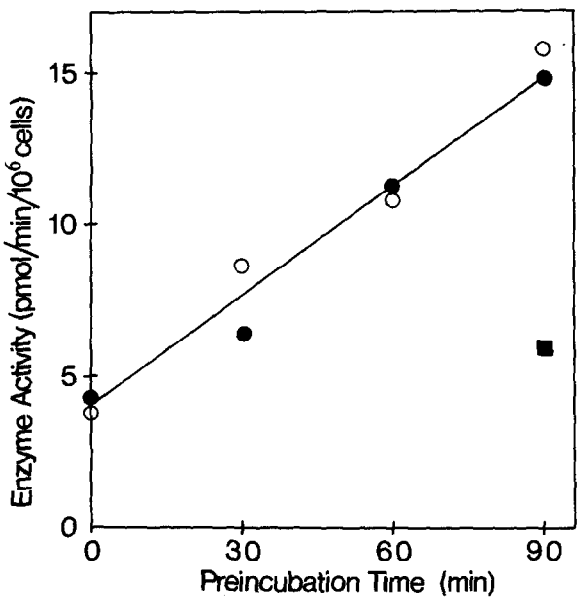

Fig.2. The enzyme activity of poly(ADP-ribose) polymerase after preincubating permeabilized cells for different times in the isotonic buffer. (•) and (○) are two separate experiments. In one experiment (▪) the permeabilized cells were preincubated for 90 min with the addition of 2 mM ATP, 100 μM each of the other seven ribo- and deoxyribonucleotides, 5 mM creatine phosphate and 100 μg/ml creatine phosphokinase.

mixture [3] prevented both the DNA fragmentation (fig.3C) and the increase in poly(ADP-ribose) polymerase activity (fig.2). We do not yet know whether we are preventing degradation of DNA in these conditions, or whether we have established an in vitro DNA repair system. In either case, it is clear that the apparent activity of poly(ADP-ribose) polymerase correlates with the size of the DNA. This was further shown in experiments where permeabilized cells were irradiated with 10 krad γ-radiation which is known to cause extensive fragmentation of DNA. This treatment increased the activity of poly(ADP-ribose) polymerase to 20 pmol/min/10^6 cells, approaching that of nuclei. These results are consistent with previous reports that deoxyribonucleases stimulate the incorporation of NAD^+ by isolated nuclei [8,9].

Although all in vitro systems necessarily differ from in vivo conditions we argue that, because their DNA is less fragmented, permeabilized cells provide a better estimation of the in vivo activity of poly(ADP-ribose) polymerase than do isolated nuclei. The maximal potential activity, however, is only realised in preparations where DNA has been fragmented either by nuclear isolation, γ-irradiation or preincubation of permeabilized cells.

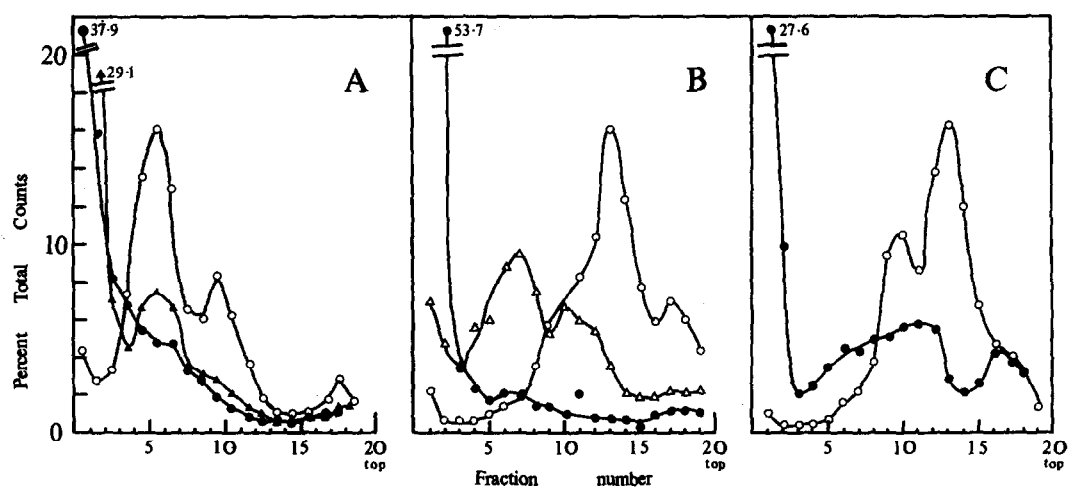

Fig.3. Alkaline sucrose gradient centrifugation. 3A: Effect of nuclear isolation and permeabilization on size of DNA: (▲) intact cells; (○) isolated nuclei; (•) permeabilized cells. In each case the preparations were treated with 500 rad γ-radiation immediately before layering onto the gradient. 3B: Effect of preincubation of permeabilized cells on DNA size: (•) no preincubation; (△) 30 min preincubation; (○) 90 min preincubation. 3C: Effect of preincubation with nucleotides on size of DNA: (○) 90 min preincubation without nucleotides; (•) 90 min preincubation with added nucleotides as described in fig.2 legend. In all three panels the top of the gradient is at the right. The vertical axis shows the percentage of total recovered counts in each fraction.

Acknowledgements

We thank our colleagues C. J. Skidmore, P. Goodwin and M. I. Davies for advice and assistance in the work and with the manuscript. This work has been supported by the Medical Research Council, the Science Research Council and the Cancer Research Campaign.

References

[1] Hilz, H. and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1–58.
[2] Hayaishi, O. and Ueda, K. (1977) Ann. Rev. Biochem. 46, 95–116.
[3] Reinhard, P., Burkhalter, M. and Gautschi, J. R. (1977) Biochim. Biophys. Acta 474, 500–511.
[4] Hunter, T. and Francke, B. (1974) J. Virol. 13, 125–139.
[5] Lehmann, A. R., Kirk-Bell, S., Shall, S. and Whish, W. J. D. (1974) Exp. Cell Res. 83, 63–72.
[6] Takakusu, A., Lazarus, H., Levine, M., McCoy, T. A. and Foley, G. E. (1968) Exp. Cell Res. 49, 226–229.
[7] Lehmann, A. R. and Ormerod, M. G. (1969) Nature 221, 1053–1056.
[8] Janakidevi, K. and Koh, C. (1974) Biochemistry 13, 1327–1330.
[9] Miller, E. G. (1975) Biochim. Biophys. Acta 395, 191–200.